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(71) Applicant: DIVERSA CORPORATION [US/US]; 10665 Sorrento Valley Road, San Diego, CA 92121 (US).

(72) Inventor: SHORT, Jay, M.; 320 Delage Drive, Encinitas, CA 92024 (US).

(74) Agent: HAILE, Lisa, A.; Gary Cary Ware & Friedenrich LLP, Suite 1600, 4365 Executive Drive, San Diego, CA 92121-2189 (US).

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(54) Title: NON-STOCHASTIC GENERATION OF GENETIC VACCINES AND ENZYMES

(57) Abstract

This invention provides methods of obtaining novel polynucleotides and encoded polypeptides by use of non-stochastic methods of directed evolution (DirectEvolution™). These methods include non-stochastic polynucleotide site-saturation mutagenesis (Gene Site Saturation Mutagenesis™) and non-stochastic polynucleotide reassembly (GeneReassembly™). Through use of the claimed methods, genetic vaccines, enzymes, and other desirable molecules can be evolved towards desirable properties. For example, vaccine vectors can be obtained that exhibit increased efficacy for use as genetic vaccines. Vectors obtained by using the methods can have, for example, enhanced antigen expression, increased uptake into a cell, increased stability in a cell, ability to tailor an immune response, and the like. This invention provides methods of obtaining novel enzymes that have optimized physical and/or biological properties. Furthermore, this invention provides methods of obtaining a variety of novel biologically active molecules, in the fields of antibiotics, pharmacotherapeutics, and transgenic traits.

Andrew G Kolomayets Esq.
Cook Alex McFarron Manzo
Cummings & Mehler Ltd
200 West Adams Street- Suite 2850
Chicago
Illinois 60606
USA

19 September 2003

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Dear Andy

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Yours sincerely

Charlotte Crowhurst PhD smj

3. CLAIMS

1. A method for obtaining an immunomodulatory polynucleotide that has an optimized modulatory effect on an immune response, or encodes a polypeptide that has an optimized modulatory effect on an immune response, the method comprising:

creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set;

wherein optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation and iterative manner;

whereby these directed evolution methods include the introduction of mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein;

and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

2. The method of claim 1, wherein said optimized modulatory effect on an immune response is induced by a genetic vaccine vector.

3. A method for obtaining an immunomodulatory polynucleotide that has an optimized modulatory effect on an immune response, or encodes a polypeptide that has an optimized modulatory effect on an immune response, the method comprising:

screening a library of non-stochastically generated progeny polynucleotides to identify an optimized non-stochastically generated progeny polynucleotide that has, or

encodes a polypeptide that has, a modulatory effect on an immune response; wherein the optimized non-stochastically generated polynucleotide or the polypeptide encoded by the non-stochastically generated polynucleotide exhibits an enhanced ability to modulate an immune response compared to a parental polynucleotide from which the library was created.

4. The method of claim 3, wherein said optimized modulatory effect on an immune response is induced by a genetic vaccine vector.

5. A method for obtaining an immunomodulatory polynucleotide that has an optimized modulatory effect on an immune response, or encodes a polypeptide that has an optimized modulatory effect on an immune response, the method comprising:

a) creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set; and

b) screening the library to identify an optimized non-stochastically generated progeny polynucleotide that has, or encodes a polypeptide that has, a modulatory effect on an immune response induced by a genetic vaccine vector; wherein the optimized non-stochastically generated polynucleotide or the polypeptide encoded by the non-stochastically generated polynucleotide exhibits an enhanced ability to modulate an immune response compared to a parental polynucleotide from which the library was created;

whereby optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation, and iterative manner;

whereby these directed evolution methods include the introduction of point mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein;

and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

6. The method of claim 5, wherein said optimized modulatory effect on an immune response is induced by a genetic vaccine vector.

7. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide is incorporated into a genetic vaccine vector.

8. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide, or a polypeptide encoded by the optimized non-stochastically generated polynucleotide, is administered in conjunction with a genetic vaccine vector.

9. The method of any of claims 1-6, wherein the library of non-stochastically generated progeny polynucleotides is created by a process selected from the group consisting of gene reassembly, oligonucleotide-directed saturation mutagenesis, and any combination, permutation and iterative manner.

10. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide that has a modulatory effect on an immune response is obtained by:

a) non-stochastically reassembling at least two parental template polynucleotide, each of which is, or encodes a molecule that is, involved in modulating an immune response;

wherein the first and second parental templates differ from each other in two or more nucleotides, to produce a library of non-stochastically generated polynucleotides; and

b) screening the library to identify at least one optimized non-stochastically generated polynucleotide that exhibits, either by itself or through the encoded molecule, an enhanced ability to modulate an immune response in comparison to a parental polynucleotide from which the library was created.

11. The method of claim 10, wherein the method further comprises the steps of:

c) subjecting a working optimized non-stochastically generated polynucleotide to a further round of non-stochastic reassembly with at least one additional polynucleotide, which is the same or different from the first and second polynucleotides, to produce a further working library of recombinant polynucleotides;

d) screening the further working library to identify at least one further optimized non-stochastically generated polynucleotide that exhibits an enhanced ability to modulate an immune response in comparison to a parental polynucleotide from which the library was created; and

e) optionally repeating c) and d) as necessary, until a desirable further optimized non-stochastically generated polynucleotide that exhibits an enhanced ability to modulate an immune response than a form of the nucleic acid from which the library was created.

12. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide encodes a polypeptide that can interact with a cellular receptor involved in mediating an immune response; wherein the polypeptide acts as an agonist or antagonist of the receptor.

13. The method of claim 12, wherein the cellular receptor is a macrophage scavenger receptor.

14. The method of claim 12, wherein the cellular receptor is selected from the group consisting of a cytokine receptor and a chemokine receptor.

15. The method of claim 14, wherein the chemokine receptor is CCR6.

16. The method of claim 12, wherein the polypeptide mimics the activity of a natural ligand for the receptor but does not induce immune reactivity to said natural ligand.

17. The method of claim 12, wherein the library is screened by:

i) expressing the non-stochastically generated progeny polynucleotides so that the encoded polypeptides are produced as fusions with a protein displayed on the surface of a replicable genetic package;

ii) contacting the replicable genetic packages with a plurality of cells that display the receptor; and

iii) identifying cells that exhibit a modulation of an immune response mediated by the receptor.

18. The method of claim 17, wherein the replicable genetic package is selected from the group consisting of a bacteriophage, a cell, a spore, and a virus.

19. The method of claim 18, wherein the replicable genetic package is an M13 bacteriophage and the protein is encoded by geneIII or geneVIII.

20. The method of claim 12, which method further comprises introducing the optimized non-stochastically generated polynucleotide into a genetic vaccine vector and administering the vector to a mammal, wherein the peptide or polypeptide is expressed and acts as an agonist or antagonist of the receptor.

21. The method of claim 12, which method further comprises producing the polypeptide encoded by the optimized non-stochastically generated polynucleotide and introducing the polypeptide into a mammal in conjunction with a genetic vaccine vector.

22. The method of claim 12, wherein the optimized non-stochastically generated polynucleotide is inserted into an antigen-encoding nucleotide sequence of a genetic vaccine vector.

23. The method of claim 22, wherein the optimized non-stochastically generated polypeptide is introduced into a nucleotide sequence that encodes an M- loop of an HBsAg polypeptide.

24. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide comprises a nucleotide sequence rich in unmethylated CpG.

25. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide encodes a polypeptide that inhibits an allergic reaction.

26. The method of claim 25, wherein the polypeptide is selected from the group consisting of interferon- , interferon- , IL- 10, IL- 12, an antagonist of IL-4, an antagonist of IL-5, and an antagonist of IL-13.

27. The method of 1, wherein the optimized recombinant polynucleotide encodes an antagonist of IL-10.

28. The method of claim 27, wherein the antagonist of IL-10 is soluble or defective IL-10 receptor or IL-20/MDA-7.

29. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide encodes a co-stimulator.

30. The method of claim 29, wherein the co-stimulator is B7-1 (CD80) or B7-2 (CD86) and the screening step involves selecting variants with altered activity through CD28 or CTLA-4.

31. The method of claim 29, wherein the co-stimulator is CD1, CD40, CD154 (ligand for CD40) or CD150 (SLAM).

32. The method of claim 29, wherein the co-stimulator is a cytokine.

33. The method of claim 32, wherein the cytokine is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL- 10, IL- 11, IL- 12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, GM-CSF, G-CSF, TNF- , IFN- , IFN- , and IL-20 (MDA-7).

34. The method of 33, wherein the library of non-stochastically generated polynucleotides is screened by testing the ability of cytokines encoded by the non-stochastically generated polynucleotides to activate cells which contain a receptor for the cytokine.

35. The method of claim 34, wherein the cells contain a heterologous nucleic acid that encodes the receptor for the cytokine.

36. The method of 33, wherein the cytokine is interleukin-12 and the screening is performed by: growing mammalian cells which contain the genetic vaccine vector in a culture medium; and detecting whether T cell proliferation or T cell differentiation is induced by contact with the culture medium.

37. The method of 33, wherein the cytokine is interferon- and the screening is performed by:

i) expressing the non-stochastically generated polynucleotides so that the encoded polypeptides are produced as fusions with a protein displayed on the surface of a replicable genetic package;

ii) contacting the replicable genetic packages with a plurality of B cells; and

iii) identifying phage library members that are capable of inhibiting proliferation of the B cells.

38. The method of claim 33, wherein the immune response of interest is differentiation of T cells to T_H1 cells and the screening is performed by contacting a population of T cells with the cytokines encoded by the members of the library of recombinant polynucleotides and identifying library members that encode a cytokine that induces the T cells to produce IL-2 and interferon- .

39. The method of claim 32, wherein the cytokine encoded by the optimized non-stochastically generated polynucleotide exhibits reduced immunogenicity compared to a

cytokine encoded by a non-optimized polynucleotide, and the reduced immunogenicity is detected by introducing a cytokine encoded by the non-stochastically generated polynucleotide into a mammal and determining whether an immune response is induced against the cytokine.

40. The method of claim 29, wherein the co-stimulator is B7-1 (CD80) or B7-2 (CD86) and the cell is tested for ability to costimulate an immune response.

41. The method of any of claims 1-6, wherein the optimized recombinant polynucleotide encodes a cytokine antagonist.

42. The method of claim 41, wherein the cytokine antagonist is selected from the group consisting of a soluble cytokine receptor and a transmembrane cytokine receptor having a defective signal sequence.

43. The method of claim 41, wherein the cytokine antagonist is selected from the group consisting of IL- 1 OR and IL-4R.

44. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide encodes a polypeptide capable of inducing a predominantly T_H1 immune response.

45. The method of any of claims 1-6, wherein the optimized non-stochastically generated polynucleotide encodes a polypeptide capable of inducing a predominantly T_H2 immune response.

46. The method of any of claims 1-6, wherein said optimized modulatory effect on an immune response is a decrease in an unwanted modulatory effect on an immune response;

whereby application of the method can be used to generate a molecule having a decreased ability to elicit an immune response from a host recipient of said molecule, where said recipient can be a human or an animal host;

and whereby application of the method can thus be used to generate a molecule having decreased antigenicity with respect to at least one host recipient of said molecule.

47. The method of any of claims 1-6, wherein said optimized modulatory effect on an immune response is an increase in a desirable modulatory effect on an immune response;

whereby application of the method can be used to generate a molecule having an increased ability to elicit an immune response from a host recipient of said molecule, where said recipient can be a human or an animal host;

and whereby application of the method can thus be used to generate a molecule having increased antigenicity with respect to at least one host recipient of said molecule.

48. The method of any of claims 1-6, wherein said optimized modulatory effect on an immune response is both a decrease in a first unwanted modulatory effect on an immune response as well as an increase in a second desirable modulatory effect on an immune response;

whereby application of the method can be used to generate a molecule having both a decreased ability to elicit a first immune response from a first host recipient of said molecule as well as a an increased ability to elicit a second immune response from a second host recipient of said molecule;

whereby the first and the second recipient hosts can be the same or different;

whereby each of the first and the second recipient hosts can be a human or an animal host;

and whereby application of the method can thus be used to generate a molecule having both a first decreased antigenicity with respect to at least one host recipient of said molecule and a second decreased antigenicity with respect to at least one host recipient of said molecule.

49. The method of claim 48, wherein said first and said second modulatory effect on an immune response are evolved for respectively a first and a second module on the same multimodule vaccine vector;

whereby a module is exemplified by the following modules, as well as by a fragment derivative or analog thereof: an antigen coding sequence, a polyadenylation sequence, a sequence coding for a co-stimulatory molecule, a sequence coding for an inducible repressor or transactivator, a eukaryotic origin or replication, a prokaryotic origin of replication, a sequence coding for a prokaryotic marker, , and enhancer, a promoter, and operator, and an intron.

50. The method of any of claims 1-6, wherein the optimized modulatory effect on an immune response is comprised of an increase in the stability of the immunomodulatory (IM) polynucleotide or polypeptide encoded thereby;

whereby application of the method can be used to generate a molecule having an increased stability ex vivo, thus, for example, increasing shelf-life and/or ease of storage and/or length of time before expiration of activity upon storage;

and whereby application of the method can also be used to generate a molecule having an increased stability in vivo upon administration to a host recipient, thus, for example, increasing resistance to digestive acids and/or increasing stability in the circulation and/or any other method of elimination or destruction by the host recipient.

51. The method of any of claims 1-6, wherein the immunomodulatory (IM) polynucleotide or polypeptide encoded thereby; has an optimized modulatory effect on an immune response in a human host recipient;

whereby application of the method can thus be used to generate an optimized genetic vaccine for human recipeints.

52. The method of any of claims 1-6, wherein the immunomodulatory (IM) polynucleotide or polypeptide encoded thereby; has an optimized modulatory effect on an immune response in an animal host recipient;

whereby application of the method can thus be used to generate an optimized genetic vaccine for animal recipients, including animals that are farmed or raised by man, animals that are not farmed or raised by man, domesticated animals, and non-domesticated animals.

53. A method for obtaining an optimized polynucleotide that encodes an accessory molecule that improves the transport or presentation of antigens by a cell, the method comprising:

a) creating a library of non-stochastically generated polynucleotides by subjecting to optimization by non-stochastic directed evolution a parental polynucleotide set in which is encoded all or part of the accessory molecule; and

b) screening the library to identify an optimized non-stochastically generated progeny polynucleotide that encodes a recombinant molecule that confers upon a cell an increased or decreased ability to transport or present an antigen on a surface of the cell compared to an accessory molecule encoded by template polynucleotides not subjected to the non-stochastic reassembly;

whereby application of the method can thus be used to generate an optimized molecule for human recipients &/or animal recipients, including animals that are farmed or raised by man, animals that are not farmed or raised by man, domesticated animals, and non-domesticated animals;

whereby optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation, and iterative manner;

whereby these directed evolution methods include the introduction of point mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein;

and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

54. The method of claim 53, wherein the screening involves:

i) introducing the library of non-stochastically generated polynucleotides into a genetic vaccine vector that encodes an antigen to form a library of vectors; introducing the library of vectors into mammalian cells; and

ii) identifying mammalian cells that exhibit increased or decreased immunogenicity to the antigen.

55. The method of claim 53, wherein the accessory molecule comprises a proteasome or a TAP polypeptide.

56. The method of claim 53, wherein the accessory molecule comprises a cytotoxic T-cell inducing sequence.

57. The method of claim 56, wherein the cytotoxic T-cell inducing sequence is obtained from a hepatitis B surface antigen.

58. The method of claim 53, wherein the accessory molecule comprises an immunogenic agonist sequence.

59. A method for obtaining an immunomodulatory polynucleotide that has, an optimized expression in a recombinant expression host, the method comprising:

creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set;

whereby optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation and iterative manner;

whereby these directed evolution methods include the introduction of mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein;

and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

60. A method for obtaining an immunomodulatory polynucleotide that has an optimized expression in a recombinant expression host, the method comprising:

screening a library of non-stochastically generated progeny polynucleotides to identify an optimized non-stochastically generated progeny polynucleotide that has an optimized expression in a recombinant expression host when compared to the expression of a parental polynucleotide from which the library was created.

61. A method for obtaining an immunomodulatory polynucleotide that has an optimized expression in a recombinant expression host, the method comprising:

a) creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set; and

b) screening a library of non-stochastically generated progeny polynucleotides to identify an optimized non-stochastically generated progeny polynucleotide that has an optimized expression in a recombinant expression host when compared to the expression of a parental polynucleotide from which the library was created;

whereby optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation, and iterative manner;

whereby these directed evolution methods include the introduction of point mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein;

and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

62. The method of any of claims 59-61, wherein the recombinant expression host is a prokaryote.

63. The method of any of claims 59-61, wherein the recombinant expression host is a eukaryote.

64. The method of claim 63, wherein the recombinant expression host is a plant.

65. The method of any of claims 64, wherein the recombinant expression host is a monocot.

66. The method of any of claims 64, wherein the recombinant expression host is a dicot.

67. The method of any of claims 1-6, 53, or 59-61, wherein creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set is comprised of subjecting the parental polynucleotide set to "gene site saturation mutagenesis" as described herein.

68. The method of any of claims 1-6, 53, or 59-61, wherein creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set is comprised of subjecting the parental polynucleotide set to "synthetic ligation polynucleotide reassembly" as described herein.

69. The method of any of claims 1-6, 53, or 59-61, wherein creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set is comprised of subjecting the parental polynucleotide set to both "gene site saturation mutagenesis" as described herein, and to "synthetic ligation polynucleotide reassembly" as described herein.

70. A method of producing a progeny polynucleotide set by subjecting a double-stranded circular parental polynucleotide molecule to mutagenesis, said method comprising the steps of:

a) annealing a first primer and a second primer to said parental polynucleotide molecule;

wherein said first primer is comprised of a first primer sequence that is complementary to a first annealment region of the parental polynucleotide molecule,

wherein said second primer is comprised of a second primer sequence that is complementary to a second annealment region of the parental polynucleotide molecule,

wherein said first annealment region and said second annealment region are non-overlapping and therefore staggered,

and wherein at least one of said first and second primers contains a non-stochastic mutagenic cassette with respect to the parental polynucleotide molecule; and

b) synthesizing by means of a polymerase-catalyzed amplification reaction a first progeny polynucleotide strand comprised of said first primer and a second progeny polynucleotide strand comprised of said second primer;

wherein the first progeny polynucleotide strand and the second progeny polynucleotide strand may form a double-stranded mutagenized circular polynucleotide product.

71. A method of producing a progeny polynucleotide set by subjecting a double-stranded circular parental polynucleotide molecule to mutagenesis, said method comprising the steps of:

a) annealing a first primer and a second primer to said parental polynucleotide molecule;

wherein said first primer is comprised of a first primer sequence that is complementary to a first annealment region of the parental polynucleotide molecule,

wherein said second primer is comprised of a second primer sequence that is complementary to a second annealment region of the parental polynucleotide molecule,

wherein said first annealment region and said second annealment region are non-overlapping and therefore staggered,

wherein at least one of said first and second primers contains a non-stochastic mutagenic cassette with respect to the parental polynucleotide molecule, and

wherein said non-stochastic mutagenic cassette contained in said at least one primer is degenerate in nature; and

b) synthesizing by means of a polymerase-catalyzed amplification reaction a first progeny polynucleotide strand comprised of said first primer and a second progeny polynucleotide strand comprised of said second primer;

wherein the first progeny polynucleotide strand and the second progeny polynucleotide strand may form a double-stranded mutagenized circular polynucleotide product;

whereby the generation of a degenerate progeny polynucleotide set may be achieved by applying said method.

72. A method for producing from a template polypeptide a set of progeny polypeptides in which a non-stochastic range of single amino acid substitutions is represented at each amino acid position, comprising the steps of:

a) subjecting a codon-containing template polynucleotide to polymerase-based amplification using a degenerate oligonucleotide for each codon to be mutagenized, wherein each of said degenerate oligonucleotides is comprised of a

first homologous sequence and a degenerate trinucleotide cassette, so as to generate a set of progeny polynucleotides; and

b) subjecting said set of progeny polynucleotides to clonal amplification such that polypeptides encoded by the progeny polynucleotides are expressed;

whereby, said method provides a means for generating a predetermined number of amino acids to be represented at each amino acid site along a parental polypeptide template, up to as many as all 20 amino acids at each of said amino acid sites.

73. The method of claim 72, wherein said degenerate oligonucleotide is comprised of a first homologous sequence, a degenerate trinucleotide cassette, and a second homologous sequence.

74. The method of claim 72, wherein said degenerate trinucleotide cassette is comprised of a first mononucleotide cassette selected from the group consisting of:

a degenerate A/C mononucleotide cassette,
a degenerate A/G mononucleotide cassette,
a degenerate A/T mononucleotide cassette,
a degenerate C/G mononucleotide cassette,
a degenerate C/T mononucleotide cassette,
a degenerate G/T mononucleotide cassette,
a degenerate C/G/T mononucleotide cassette,
a degenerate A/G/T mononucleotide cassette,
a degenerate A/C/T mononucleotide cassette,
a degenerate A/C/G mononucleotide cassette,
and a degenerate N or A/C/G/T mononucleotide cassette;

and wherein said degenerate trinucleotide cassette is further comprised of a second and a third mononucleotide cassette, each selected from the group consisting of:

a degenerate A/C mononucleotide cassette,
a degenerate A/G mononucleotide cassette,
a degenerate A/T mononucleotide cassette,
a degenerate C/G mononucleotide cassette,
a degenerate C/T mononucleotide cassette,
a degenerate G/T mononucleotide cassette,
a degenerate C/G/T mononucleotide cassette
a degenerate A/G/T mononucleotide cassette,
a degenerate A/C/T mononucleotide cassette,
a degenerate A/C/G mononucleotide cassette,
a degenerate N or A/C/G/T mononucleotide cassette,
a non-degenerate A mononucleotide cassette,
a non-degenerate C mononucleotide cassette,
a non-degenerate G mononucleotide cassette,
and a non-degenerate T mononucleotide cassette.

75. The method of claim 72, where said degenerate trinucleotide cassette is selected from the group consisting of:

a degenerate N,N,N trinucleotide cassette,
a degenerate N,N,G/T trinucleotide cassette,
a degenerate N,N,G/C trinucleotide cassette,
a degenerate N,N,A/C/G trinucleotide cassette,
a degenerate N,N,A/G/T trinucleotide cassette,
and a degenerate N,N,C/G/T trinucleotide cassette;

whereby, said method provides a means for generating all 20 amino acid changes at each amino acid site along a parental polypeptide template, because the degeneracy of the specified trinucleotide cassette sequences includes codons for all 20 amino acids.

76. The method of claim 72, wherein said degenerate oligonucleotide is comprised of a first homologous sequence and a plurality of trinucleotide cassettes;

whereby, said method provides a means for generating a progeny polypeptide having a plurality of concurrent single amino acid changes with respect to a parental polypeptide template.

77. The method of claim 76, wherein each of said degenerate trinucleotide cassettes is comprised of a first mononucleotide cassette selected from the group consisting of:

a degenerate A/C mononucleotide cassette,
a degenerate A/G mononucleotide cassette,
a degenerate A/T mononucleotide cassette,
a degenerate C/G mononucleotide cassette,
a degenerate C/T mononucleotide cassette,
a degenerate G/T mononucleotide cassette,
a degenerate C/G/T mononucleotide cassette,
a degenerate A/G/T mononucleotide cassette,
a degenerate A/C/T mononucleotide cassette,
a degenerate A/C/G mononucleotide cassette,

and a degenerate N or A/C/G/T mononucleotide cassette;

and wherein each of said degenerate trinucleotide cassettes is further comprised of a second and a third mononucleotide cassette, each selected from the group of consisting of:

a degenerate A/C mononucleotide cassette,
a degenerate A/G mononucleotide cassette,
a degenerate A/T mononucleotide cassette,
a degenerate C/G mononucleotide cassette,
a degenerate C/T mononucleotide cassette,
a degenerate G/T mononucleotide cassette,
a degenerate C/G/T mononucleotide cassette
a degenerate A/G/T mononucleotide cassette,
a degenerate A/C/T mononucleotide cassette,
a degenerate A/C/G mononucleotide cassette,
a degenerate N or A/C/G/T mononucleotide cassette,
a non-degenerate A mononucleotide cassette,
a non-degenerate C mononucleotide cassette,
a non-degenerate G mononucleotide cassette,
and a non-degenerate T mononucleotide cassette.

78. The method of claim 76, where said degenerate trinucleotide cassette is selected from the group consisting of:

a degenerate N,N,N trinucleotide cassette,
a degenerate N,N,G/T trinucleotide cassette,
a degenerate N,N,G/C trinucleotide cassette,
a degenerate N,N,A/C/G trinucleotide cassette,

a degenerate N,N,A/G/T trinucleotide cassette,
and a degenerate N,N,C/G/T trinucleotide cassette;

whereby, said method provides a means for generating all 20 amino acid changes at each amino acid site along a parental polypeptide template, because the degeneracy of the specified trinucleotide cassette sequences includes codons for all 20 amino acids.

79. The method of claim 72, wherein said degenerate oligonucleotide is comprised of a first homologous sequence, and a plurality of trinucleotide cassettes, and a second homologous sequence.

80. A method for producing from a template polypeptide a set of progeny polypeptides in which a non-stochastic range of single amino acid substitutions is represented at each amino acid position, and for identifying desirable amino acid substitutions and combinations thereof among the progeny molecules, comprising the steps of:

a) subjecting a codon-containing template polynucleotide to polymerase-based amplification using a degenerate oligonucleotide cassette for each codon to be mutagenized, wherein each of said degenerate oligonucleotides is comprised of a first homologous sequence and a degenerate trinucleotide cassette, so as to generate a set of progeny polynucleotides; and

b) subjecting said set of progeny polynucleotides to clonal amplification such that polypeptides encoded by the progeny polynucleotides are expressed; and

c) subjecting said expressed progeny polypeptides to screening in order to compare them to the parental polynucleotide with respect to at least one molecular property of interest;

whereby, said method provides a means for generating a predetermined number of amino acids to be represented at each amino acid site along a parental polypeptide template, up to as many as all 20 amino acids at each of said amino acid sites; and

whereby, said method provides a means for identifying among said progeny polypeptides those that display a desirable change with respect to at least one molecular property when compared with its parental polypeptide.

81. The method of claim 80, wherein said degenerate trinucleotide cassette is comprised of a first nucleotide selected from the group consisting of:

a degenerate A/C mononucleotide cassette,
a degenerate A/G mononucleotide cassette,
a degenerate A/T mononucleotide cassette,
a degenerate C/G mononucleotide cassette,
a degenerate C/T mononucleotide cassette,
a degenerate G/T mononucleotide cassette,
a degenerate C/G/T mononucleotide cassette,
a degenerate A/G/T mononucleotide cassette,
a degenerate A/C/T mononucleotide cassette,
a degenerate A/C/G mononucleotide cassette,
and a degenerate N or A/C/G/T mononucleotide cassette;

and wherein said degenerate trinucleotide cassette is further comprised of a second and a third mononucleotide cassette, each selected from the group consisting of:

a degenerate A/C mononucleotide cassette,
a degenerate A/G mononucleotide cassette,
a degenerate A/T mononucleotide cassette,
a degenerate C/G mononucleotide cassette,
a degenerate C/T mononucleotide cassette,
a degenerate G/T mononucleotide cassette,
a degenerate C/G/T mononucleotide cassette
a degenerate A/G/T mononucleotide cassette,
a degenerate A/C/T mononucleotide cassette,
a degenerate A/C/G mononucleotide cassette,
a degenerate N or A/C/G/T mononucleotide cassette,
a non-degenerate A mononucleotide cassette,
a non-degenerate C mononucleotide cassette,
a non-degenerate G mononucleotide cassette,
and a non-degenerate T mononucleotide cassette.

82. The method of claim 80, where said degenerate trinucleotide cassette is selected from the group consisting of:

a degenerate N,N,N trinucleotide cassette,
a degenerate N,N,G/T trinucleotide cassette,
a degenerate N,N,G/C trinucleotide cassette,
a degenerate N,N,A/C/G trinucleotide cassette,
a degenerate N,N,A/G/T trinucleotide cassette,
and a degenerate N,N,C/G/T trinucleotide cassette;

whereby, said method provides a means for generating all 20 amino acid changes at each amino acid site along a parental polypeptide template, because the

degeneracy of the specified trinucleotide cassette sequences includes codons for all 20 amino acids.

83. The method of claim 80, wherein said degenerate oligonucleotide is comprised of a first homologous sequence and a plurality of trinucleotide cassettes;

whereby, said method provides a means for generating a progeny polypeptide having a plurality of concurrent single amino acid changes with respect to a parental polypeptide template.

84. The method of claim 80, wherein each of said degenerate trinucleotide cassettes is comprised of a first mononucleotide cassette selected from the group consisting of:

a degenerate A/C mononucleotide cassette,
a degenerate A/G mononucleotide cassette,
a degenerate A/T mononucleotide cassette,
a degenerate C/G mononucleotide cassette,
a degenerate C/T mononucleotide cassette,
a degenerate G/T mononucleotide cassette,
a degenerate C/G/T mononucleotide cassette,
a degenerate A/G/T mononucleotide cassette,
a degenerate A/C/T mononucleotide cassette,
a degenerate A/C/G mononucleotide cassette,
and a degenerate N or A/C/G/T mononucleotide cassette;

and wherein each of said degenerate trinucleotide cassettes is further comprised of a second and a third mononucleotide cassette, each selected from the group consisting of:

a degenerate A/C mononucleotide cassette,

a degenerate A/G mononucleotide cassette,

a degenerate A/T mononucleotide cassette,

a degenerate C/G mononucleotide cassette,

a degenerate C/T mononucleotide cassette,

a degenerate G/T mononucleotide cassette,

a degenerate C/G/T mononucleotide cassette

a degenerate A/G/T mononucleotide cassette,

a degenerate A/C/T mononucleotide cassette,

a degenerate A/C/G mononucleotide cassette,

a degenerate N or A/C/G/T mononucleotide cassette,

a non-degenerate A mononucleotide cassette,

a non-degenerate C mononucleotide cassette,

a non-degenerate G mononucleotide cassette,

and a non-degenerate T mononucleotide cassette.

85. The method of claim 80, where said degenerate trinucleotide cassette is selected from the group consisting of:

a degenerate N,N,N trinucleotide cassette,
a degenerate N,N,G/T trinucleotide cassette,
a degenerate N,N,G/C trinucleotide cassette,
a degenerate N,N,A/C/G trinucleotide cassette,
a degenerate N,N,A/G/T trinucleotide cassette,
and a degenerate N,N,C/G/T trinucleotide cassette;

whereby, said method provides a means for generating all 20 amino acid changes at each amino acid site along a parental polypeptide template, because the degeneracy of the specified trinucleotide cassette sequences includes codons for all 20 amino acids.